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(54) Title: NON-TOXIX AMOUNTS OF PROTEIN-AGGREGATING SUBSTANCES STIMULATE HSP70 EXPRESSION AND FUNCTION AS ANTI-TUMOR AGENTS

(57) Abstract: The present invention relates to the use of a drug inducing intracellular protein aggregation for the preparation of a pharmaceutical composition wherein the drug is in a dose of 135-175 mg/m2 body surface for the treatment of a tumor, a bacterial infection or a viral infection. Preferably said drug is selected from vincristine and paclitaxel. The present invention further relates to a method, of treating a patient suffering from a tumor, a bacterial infection or a viral infection comprising administering to said patient a drug inducing intracellular protein aggregation in a dose of 135-175 mg/m^2 body surface. It is preferred that said drug is administered in combination with heat treatment.

Non-toxic amounts of protein-aggregating substances stimulate Hsp70 expression and function as anti-tumor agents

The present invention relates to the use of a drug inducing intracellular protein aggregation for the preparation of a pharmaceutical composition wherein the drug is in a dose of 135-175 mg/m^2 body surface for the treatment of a tumor, a bacterial infection or a viral infection. Preferably said drug is selected from vincristine and paclitaxel. The present invention further relates to a method of treating a patient suffering from a tumor, a bacterial infection or a viral infection comprising administering to said patient a drug inducing intracellular protein aggregation in a dose of 135-175 mg/m^2 body surface. It is preferred that said drug is administered in combination with heat treatment.

The specification recites a number of prior art documents. The disclosure content of these documents including manufacturer's manuals, is herewith incorporated by reference.

Following physical as well as chemical stress the synthesis of heat shock proteins (HSP) is induced in prokaryotic as well as eukaryotic cells in order to protect the cells from lethal damage. Under physiological conditions (37°C) constitutively expressed HSP fulfill important functions such as chaperoning proteins during synthesis, folding, transport, assembly, and protein degradation (Hartl 1996; Csermely 2001). Hsp70, the major stress-inducible member of the HSP70 family, translocates from the cytoplasm to the nucleus following stress. Beside their chaperoning function, HSP70s are known to play important roles in cancer immunity either as carrier proteins for tumor-specific peptides (Blachere *et al.* 1997), as inducers for proinflammatory cytokines (Asea *et al.* 2000), or as stimulators for NK activity (Multhoff *et al.* 1999). Recently, a receptor-mediated uptake has been described for HSP-peptide complexes into antigen presenting cells (APC). HSP-chaperoned tumor-derived peptides are re-presented on major histocompatibility antigen complex (MHC) class I

complexes into antigen presenting cells (APC). HSP-chaperoned tumor-derived peptides are re-presented on major histocompatibility antigen complex (MHC) class I molecules, and thus elicit a CD8 mediated T cell immune response (Basu *et al.* 2001; Binder *et al.* 2000; Nakayama *et al.* 1999). An unusual plasma membrane localization of Hsp70 was detected that correlates with the sensitivity of tumor cells to lysis mediated by natural killer (NK) cells (Multhoff *et al.* 1995a; Multhoff *et al.* 1995b; Multhoff *et al.* 1997). Furthermore, it was demonstrated that physical (i.e. heat), as well as chemical (i.e. ET-18-OCH_3) stress selectively increases the amount of membrane-bound Hsp70 on tumor cells, but not on normal cells (Botzler *et al.* 1999) and thus renders them better targets for NK lysis.

In contrast to these findings that demonstrate an immunostimulatory capacity of Hsp70, high cytoplasmic Hsp70 levels have been reported to contribute to anti-apoptotic mechanisms in different tumor cell systems (Wei *et al.* 1994; Gabai *et al.* 1998; Jäättelä *et al.* 1998). Because of this dual activity of Hsp70, it is important to study not only the amount, but also the cellular and subcellular localization of Hsp70, to understand its specific immune function. High intracellular Hsp70 levels in prostate carcinoma cells are associated with drug resistance (Roigas *et al.* 1998).

Cytarabine and ifosfamide are potent antineoplastic drugs with a broad spectrum of biological activities. Both compounds affect replicating cells and interact with DNA. Cytarabine, a pyrimidine antagonist that is converted in arabinosyl-cytosintriphosphat by kinases, is most efficient in the S phase of the cell cycle and therefore predominantly affects viability of rapidly growing cells (Grant 1998). Cytarabine undergoes phosphorylation by deoxycytidine kinases before its incorporation into DNA, which finally results in cell death. Clinically, cytarabine is applied in the treatment of acute lymphoblastic leukemia (ALL), Non-Hodgkin lymphoma (NHL) and acute myelogenous leukemia (AML). The oxazaphosphorine ifosfamide is most frequently used for the treatment of solid tumors. *In vivo*, ifosfamide is metabolically activated by hepatic mixed-function oxidases into 4-hydroxyifosfamide (4-OH-IF) which is decomposed to alkylating mustard (Zalupski and Baker 1988; Multhoff *et al.* 1995c). The DNA-alkylating and crosslinking activity of the mustard induces cell death especially in proliferating cells. The remaining metabolites are acrolein,

aldocyclophosphamide/ aldoifosfamide and chloroacetaldehyde which are produced alternatively by beta-oxidation form conjugates with glutathione. In order to mimick the metabolically active form of ifosfamide, for *in vitro* investigations the prodrug 4-hydroperoxyifosfamide (4-OOH-IF) was used, which in aqueous solutions rapidly gives rise to pharmacologically equivalent amounts of the activated form of ifosfamide (4-OH-IF). Vincristine is used either as a single agent or in combination with ifosfamide, methotrexate, and cytarabine in a number of solid tumors and in hematological malignancies including ALL, Acute Non-Lymphoblastic Leukemia (ANLL) or Non-Hodgkin Lymphoma (NHL). Vincristine is known to bind microtubular proteins, inhibits the mitotic spindle formation and thus causes an arrest in the metaphase of mitosis (Gidding *et al.* 1999). Paclitaxel, the effective compound of Taxol-100 is used for the treatment of ovarian and mammary carcinomas (Eisenhauer *et al.* 1998). It is assumed that paclitaxel binds to tubulin dimers and thus disables the dynamic reorganization of the tubular network during the active interphase and during mitosis. It supports the formation of microtubuli aggregates and inhibits their depolymerization (Kingston 2000; Snyder *et al.* 2001).

A significant disadvantage of the state of the art cancer treatment using any of the above recited anti-tumor drugs is the recognized necessity to administer said drugs in relatively high concentrations. These high concentrations are *in vivo* toxic not only for tumor cells but also for non-tumorous cells. As a consequence, a patient treated with the prescribed regimen of any of those drugs suffers from severe adverse effects. Similar problems with severe side effects are often encountered in the treatment of bacterial or viral infections.

In view of the above, the technical problem underlying the present invention was to provide means and methods for an essentially efficacious treatment, in particular of tumors but also of bacterial or viral infections that is not accompanied by said severe adverse effects.

The solution to said technical problem is achieved by providing the embodiments characterized in the claims.

Thus, the present invention relates to the use of a drug inducing intracellular protein aggregation for the preparation of a pharmaceutical composition wherein the drug is in a dose of 135-175 mg/m^2 body surface for the treatment of a tumor, a bacterial or viral infection.

In accordance with the present invention, the term "intracellular protein aggregates" is intended to mean an aggregate of proteins that does not normally or under physiological conditions occur in a viable, non-tumorous cell. However, Hsp70 is induced by vincristine (V) and paclitaxel (P) in the cytoplasma of normal cells (a corresponding experiment is shown in Fig 2C). Such aggregates may consists of the same as well as of different proteins. Generally, such protein aggregates may be formed upon the application of stress, e.g. in the form of heat, radiation or chemicals, to a cell. The formation of said aggregate eventually renders the cell bound to cell death. This is because it was found in accordance with the present invention that drugs administered in the above indicated range to a patient, preferably a human, and inducing protein aggregation will induce Hsp70 to migrate to the tumor cell surface or to the cell surface of the infected cell where it functions as a target structure for Natural Killer (NK) cells. In contrast HSP70 cell surface expression is not induced in healthy cells, i.e. PBL (a corresponding experiment is shown in Fig 4B).

The teaching of the present invention therefore provide a much improved approach to the treatment of a large variety of tumors or of bacterial or viral infections without the severe adverse effects that were concomitantly observed with the prior art drug anti-cancer etc. treatment. It is to be noted that the concentration range of the drugs is in accordance with the present invention, clearly a range sublethal for normal cells.

Since the effect on tumor or infected cells is the exposure of Hsp70 on their surface, the present invention may also be viewed as the use of drug inducing exposure of Hsp70 on the surface of tumor or infected cells for the preparation of a pharmaceutical composition wherein the drug is in a dose of 135 – 175 mg/m^2 body surface for the treatment of a tumor or of bacterially or virally infected cells. Viruses include HIV such as HIV I and HIV II as well as adenovirus. Further viruses include EBV (Epstein-Barr Virus), CMV (Cytomegalivirus), Coxsachievirus, Herpes simplex

virus, HPV (human papilloma virus) and Human T-cell leukemia virus. Bacteria include Mycobacterium tuberculosis and Listeria.

Based on the teachings of the present invention, the person skilled in the art is easily in a position to identify drugs in addition to those specifically disclosed in the present specification that will induce intracellular protein aggregation resulting in the specific elimination of tumor cells.

For example, protein aggregation in cells may be assessed by means of an anti-tubulin antibody that is fluorescently labeled. Aggregates of tubulin are detected as clumps on the basis of the fluorescence intensity and using, for example, a fluorescence microscope. On the other hand, the occurrence of Hsp70 on the cell surface of tumor cells may be measured using the technology described in the appended examples. One convenient method to assess Hsp70 occurrence as density on the surface of tumor cells or infected cells is flow cytometry.

The term "inducing intracellular protein aggregation" is intended to mean that the drug, upon administration, is causative for the onset or occurrence of protein aggregation. The drug may directly induce aggregation by acting on the proteins that form the aggregate. Alternatively, the drug may have an indirect effect on the onset or occurrence of protein aggregation. For example, the drug may interact with a protein in a signal cascade wherein the signal cascade eventually leads to protein aggregation.

The "pharmaceutical composition" prepared in accordance with the present invention may further comprise a pharmaceutically acceptable carrier and/or diluent. Examples of suitable pharmaceutical carriers are well known in the art and include phosphate buffered saline solutions, water, emulsions, such as oil/water emulsions, various types of wetting agents, sterile solutions etc. Compositions comprising such carriers can be formulated by well known conventional methods. These pharmaceutical compositions can be administered to the subject at a suitable dose. Administration of the suitable compositions may be effected by different ways, e.g., by intravenous,

intraperitoneal, subcutaneous, intramuscular, topical, intradermal, intranasal or intrabronchial administration. The exact dosage regimen will be determined by the attending physician and clinical factors. As is well known in the medical arts, dosages for any one patient depends upon many factors, including the patient's size, body surface area, age, the particular compound to be administered, sex, time and route of administration, general health, and other drugs being administered concurrently. The compositions of the invention may be administered locally or systemically. Administration will generally be parenterally, e.g., intravenously. Preparations for parenteral administration include sterile aqueous or non-aqueous solutions, suspensions, and emulsions. Examples of non-aqueous solvents are propylene glycol, polyethylene glycol, vegetable oils such as olive oil, and injectable organic esters such as ethyl oleate. Aqueous carriers include water, alcoholic/aqueous solutions, emulsions or suspensions, including saline and buffered media. Parenteral vehicles include sodium chloride solution, Ringer's dextrose, dextrose and sodium chloride, lactated Ringer's, or fixed oils. Intravenous vehicles include fluid and nutrient replenishers, electrolyte replenishers (such as those based on Ringer's dextrose), and the like. Preservatives and other additives may also be present such as, for example, antimicrobials, anti-oxidants, chelating agents, and inert gases and the like.

The term "wherein the drug is in a dose of 135-175 mg/m^2 body surface" describes the dosage of active ingredient required in accordance with the present invention. It is important to note that this dosage regimen is non-toxic for non-tumorous or non-infected cells. This dose corresponds to a serum content of 10 μM or lower. The person skilled in the art is easily in a position to transform this value into a different commonly used prescription of the dose, e.g. in a dose calculated based on the amount of drug (in milligrams) to be administered per kilogram of body weight. The claimed invention specifically includes embodiments wherein the prescribed dosage is administered is administered more than once, such as two, there, four, five, six, seven, eight, nine, ten or more times.

In accordance with the invention, it was found that one dose is already effective in the induction of surface exposition of Hsp70 by tumor cells or infected cells.

Accordingly, a clinical effect is already observed after a single dose treatment. Administration of multiple doses will, as a rule, enhance the clinical effect.

The term "tumor" includes malignant as well as non-malignant tumors. A malignant tumor differs from a non-malignant tumor by its ability to invade surrounding tissues.

Preferably, the drug is an anti-cancer drug, i.e. a drug that was known in the art to selectively kill tumor cells and preferably malignant tumor cells.

It is further preferred that said drug binds to microtubuli and interferes with the progress of mitosis. Accordingly, it is also preferred that said drug induces the formation of tubulin aggregates. The present invention demonstrates the capacity of antitumor agents that induce protein aggregation in cells to induce Hsp70, the major heat-inducible member of the 70kDa heat shock protein family. From the prior art, it was known that on the one hand, elevated levels of Hsp70 are discussed to play a crucial role in the induction of anti-apoptotic mechanisms that enable tumor cells to escape from apoptotic cell death induced by stimuli including heat shock, tumor necrosis factor, oxidative stress, cytostatic drugs, and radiation (Roigas *et al.* 1998; Li *et al.* 2000; Jäättelä *et al.* 1998; Ricci *et al.* 2001). Insofar the results obtained in accordance with the present invention must be viewed as surprising. On the other hand, extracellular Hsp70 induces NK activity (Multhoff *et al.* 1999) and plasma membrane-bound Hsp70 has been determined as a tumor-selective target recognition structure for NK cells (Multhoff *et al.* 1997). A stress-inducible (heat or ET-18-OCH3) increased amount of membrane-bound Hsp70 renders tumor cells more sensitive to the lysis mediated by NK cells (Botzler *et al.* 1999; Multhoff *et al.* 1995c). This dual activity of Hsp70 is dependent on the subcellular distribution of Hsp70. Also surprising was that the known anti-tumor agents may be employed for the treatment of bacterial or viral infections.

In accordance with the present invention it was also addressed whether enhanced cytoplasmic Hsp70 levels correlate with an increased amount of Hsp70 in the plasma membrane. Previously, it has been shown that nonlethal heat and nontoxic concentrations of the alkyl-lysophospholipid derivative ET-18-OCH3 are able to

stimulate an increase in plasma membrane-bound Hsp70 in K562 cells, but not in normal cells (Botzler *et al.* 1999). As already mentioned, the quantity of membrane-bound Hsp70 is associated with an enhanced NK cell mediated killing activity against tumor cells (Multhoff *et al.* 1997; Multhoff *et al.* 1995c). Furthermore, Hsp70 was not only found on the cell surface of tumor cell lines but also on freshly isolated biopsy material of carcinoma and leukemic patients (Hantschel *et al.* 2000).

Interestingly, following incubation of K562 cells (ATCC CCL 243) with nontoxic doses of drugs inducing intracellular protein aggregation such as vincristine or paclitaxel not only induce increased cytoplasmic but also membrane-bound Hsp70 levels. In contrast, the antitumor agents that interact with DNA neither increase the amount of cytoplasmic nor enhance the amount of membrane-bound Hsp70 in tumor cells (see appended examples). This is in agreement with the idea of Hightower that Hsp70 induction is a sensitive marker for proteotoxicity rather than genotoxicity (Hightower 1991).

It is important to note that Hsp70 membrane localization was restricted to tumor cells or infected cells; PBL derived from healthy human individuals did not exhibit any cell surface localization either under physiological conditions or following stress (Multhoff *et al.* 1995a, 1995b). In accordance with the present invention, it was demonstrated that treatment with heat alone or with heat plus tubulin interacting agents also did not increase membrane-bound Hsp70 on PBL. This indicated that Hsp70 membrane localization induced by vincristine or paclitaxel is tumor cell specific or specific for cells infected by bacteria or viruses. The immunological consequences of increased amounts of membrane-bound Hsp70 were shown for various tumors including human colon carcinoma cells and K562 cells. In accordance with the invention, it could be demonstrated that the sensitivity to lysis mediated by NK cells was drastically increased following incubation with the above-mentioned drug type and in particular with a nontoxic dose of paclitaxel.

The findings of the present invention are particularly advantageous since they allow the treatment of patients suffering from tumors or bacterial or viral infections with doses of medicaments that are clearly below the level of toxicity required for the killing of non-tumorous or non-infected cells. For example, the non-toxic value for

vincristine is between 0 and 250μM when tested in vitro with K562 cells; see Figure 6 and Table 2. Using the same test system, the value for paclitaxel is between 0 and 10μM, see Figure 7 and Table 2. The range in which the drugs stimulate Hsp70 surface expression is marked by bars. It is further advantageous in accordance with the present invention that the phenomenon of intracellular protein aggregation could be linked with the fact that tumor cells are susceptible to the pharmaceological effects of anti-cancer drugs administered at a much lower dose than prescribed in state of the art anti-cancer pharmaceutical compositions. Insofar, the person skilled in the art may recur to further anti-cancer drugs as drugs effective in the treatment of viral or bacterial infections having the same effects on intracellular protein aggregation and not exemplified in this specification with the sound expectation that these drugs efficaciously eradicate tumor or infected cells when administered at non-toxic levels. In a further aspect of the invention, the dosages of the drug deviate from the range indicated above but are nevertheless in a non-toxic range. Another advantage of the present invention is that many of the drugs that fall under the scope of this invention have been approved and registered for essentially the same indications that are followed up here.

Accordingly, the use of said drug in the preparation of a pharmaceutical composition for he treatment of a tumor or a bacterial or viral infection wherein the drug yields plasma levels after administration of 5 to 10μM and induces intracellular protein aggregation or cell surface expression of Hsp70 is another preferred embodiment of the invention.

The administration of the drug in the above indicated range will yield plasma levels between 5 and 10 μM. In vitro, the drugs can be tested in appropriate systems in ranges essentially between 1 nM and 300 μM wherein levels of 10 nM to 10 μM (for paclitaxel) or 10nM to 250μM (for vincristine) indicate pharmacological activity as well as non-toxicity of the drugs.

In a preferred embodiment of the use of the present invention said antineoplastic drug is vincristine.

Vincristine targets ß-tubulin and thus results in tubulin assembly. Vincristine supports the assembly of tubulin and thus causes protein aggregation within the cell (Gidding *et al.* 1999);

It is further preferred that the drug administered is Vincristin Liquid, the clinically applied homologue of vincristine. Vincristine Liquid is manufactured and distributed by Eli Lilly.

In a further preferred embodiment of the use of the present invention said drug is paclitaxel.

Just as vincristine, paclitaxel targets β-tubulin and thus induces tubulin assembly. Further, paclitaxel blocks mitosis by binding and stabilizing microtubules (Schiff and Horwitz 1980).

It is also preferred in accordance with the present invention that said drug is Taxol®-100, the clinically applied homologue of paclitaxel. Taxol®-100 is manufactured and distributed by Bristol Myers Squibb.

As demonstrated by immunostaining, vincristine as well as paclitaxel at nonlethal concentrations both induced tubulin aggregates, whereas DNA-interacting agents failed to do so.

The tumor treated in accordance with the present invention may be a benign tumor.

In an additionally preferred embodiment of the use of the present invention said tumor is a malign tumor. The malign tumor may either be a solid tumor or a hematological malignancy.

In a particularly preferred embodiment of the use of the present invention said malign tumor is a tumor selected from the following: Colorectal, pancreas, melanoma, lung carcinoma (small and non-small), ovarian, kidney, mammary, head and neck, stomach, liver, oesophagus, prostata carcinomas, and sarcomas.

It is also preferred that said hematological malignancy is selected from myeloproliferative diseases, non-Hodgkin's lymphoma (NHL), AML (acute myelocytic

leukemia), acute lymphocytic leukemia (ALL), multiple myeloma, chronic myelocytic leukemia (CML) and chronic lymphocytic leukemia (CLL), B- and T-lymphomas, CNS lymphomas, gastrointestinal lymphomas, and cutaneous lymphomas.

The present invention furthermore relates to a method of treating a patient suffering from a tumor or a bacterial or viral infection comprising administering to said patient a drug inducing intracellular protein aggregation in a dose of 135-175mg/m^2 body surface.

As regards the definitions, comments, elaborations on this embodiment of the invention and the following preferred embodiments thereof, reference is made to the foregoing.

In a preferred embodiment of the method of the present invention said drug is vincristine.

In an additionally preferred embodiment of the method of the present invention said drug is paclitaxel.

In a further preferred embodiment of the method of the present invention said tumor is a malign tumor.

In a particularly preferred embodiment of the method of the present invention said malign tumor is a tumor selected from the following: Colorectal, pancreas, melanoma, lung carcinoma (small and non-small), ovarian, kidney, mammary, head and neck, stomach, liver, oesophagus, prostata carcinomas, and sarcomas.

It is also preferred that said hematological malignancy is selected from myeloproliferative diseases, non-Hodgkin's lymphoma (NHL), AML (acute myelocytic leukemia), acute lymphocytic leukemia (ALL), multiple myeloma, chronic myelocytic leukemia (CML) and chronic lymphocytic leukemia (CLL), B- and T-lymphomas, CNS lymphomas, gastrointestinal lymphomas, and cutaneous lymphomas.

In another preferred embodiment of the present invention the method further comprises the administration of heat in the range between > 37°C to 43°C for 1 to 12

hours to said patient. Thus, heat is also administered in a sublethal dose, also when administered in conjunction with the drug.

This embodiment of the present invention is particularly advantageous due to the fact that an additive effect could be observed when heat was administered in addition to the drug. The heat may be administered simultaneously, or it may be administered prior to administration of the drug. However, care needs to be taken that there is a time overlap of the pharmacological effect due to the application of the drug and the heat treatment. Generally, if the patient is treated at a higher temperature, the time of heat administration may be shorter than if the patent is treated at a low temperature. It must be emphasized, however, that the general range of the drug goes well with the indicated heat range. A preferred range is 38°C to 41°C. Heat may be administered to a certain part of the patient's body or a whole body by hyper thermal treatment. Sources of heat includes microwaves (companies distributing appropriate instruments include BSD, Sennewald, Sigma Eye, Siemens) infrared radiation, and hot-air.

Finally, the present invention relates to a pharmaceutical composition comprising a drug inducing intracellular protein aggregation in a dose of 135-175 mg/m^2 body surface.

Preferably, the drug interacts with tubulin, as has been described above. It is further preferred that the drug is vincristine or paclitaxel (particularly Vincristin Liquid or Taxol®-100). The pharmaceutical composition may comprise one or more doses of the drug to be formulated and administered as described above.

The figures show:

Fig 1.

Influence of different antineoplastic agents on the induction of apoptosis in untreated (37°C) and heat-shocked (41.8°C) K562 cells. The percentage of apoptotic cells was determined following Annexin-FITC staining by flow cytometric analysis.

A) Apoptosis was determined 24 hours following incubation of K562 cells with the nonlethal concentration 1μM cytarabine (C), the activated form of ifosfamide (I), vincristine (V), or paclitaxel (P).

B) As a positive control for apoptosis, K562 cells were incubated with Camptothecin (Campto) at a concentration of 2μg/ ml.

Fig 2.

Influence of antineoplastic drugs on the cytoplasmic amount of Hsp70 in K562 cells. Equal protein amounts were run on a 10% SDS-PAGE and transferred to nitrocellulose membranes. Following Western blotting using the Hsp70 specific MoAb the immunoblots were quantified by densitometry. The inset represents one representative experiment out of three.

A) Representative Hsp70 immunoblots of K562 cells (37°C) incubated with no (ctrl), 10nM, 1μM, 100μM of cytarabine (C), the activated form of ifosfamide (I), vincristine (V), or paclitaxel (P).

B) Representative Hsp70 immunoblots of K562 cells following nonlethal heat shock (41.8°C) incubated with no (control), 10nM, 1μM, 100μM of cytarabine (C), the activated form of ifosfamide (I), vincristine (V), or paclitaxel (P).

The relative increase of cytoplasmic Hsp70 is indicated for each antineoplastic agent graphically and as numbers (fold increase) on the right hand side.

C) Cytoplasmic Hsp70 levels were increased in PBL of healthy donors following treatment with vincristine (V) or paclitaxel (P) with or without heat.

Representative Hsp70 immunoblots of peripheral blood lymphocytes (PBL), either under physiological temperatures (37°C) or following non-lethal heat shock (41.8°C) incubated without (ctrl) orwith 1 μM paclitaxel (P).

Fig 3.

Influence of antineoplastic drugs on membrane-bound Hsp70 in K562 cells.

Equal protein amounts of plasma membrane fractions of K562 cells (37°C) or following nonlethal heat shock (41.8°C) were run on a 10% SDS-PAGE and transferred to nitrocellulose membranes. Western blotting was performed using the Hsp70 specific MoAb.

A) Representative Hsp70 immunoblots of untreated (37°C) or heat-shocked (41.8°C)

K562 membrane fractions following incubation with 1μM of the cytostatic drugs cytarabine (C), activated form of ifosfamide (I), vincristine (V), and paclitaxel (P). Before isolation of plasma membranes K562 cells were either kept under physiological conditions or heat-shocked at the nonlethal temperature of 41.8°C.

B) Quantitative analysis of the immunoblots by densitometry. The relative increase of membrane-bound Hsp70 is indicated for each antineoplastic agent graphically and as numbers (fold increase) on the right hand side.

Fig 4.

A) Flow cytometric analysis of membrane-bound Hsp70 on K562 tumor cells.
Untreated (37°C) or heat-shocked (41.8°C) K562 cells were incubated with 1μM of the cytostatic drugs vincristine (V) or paclitaxel (P) and stained with a FITC-conjugated Hsp70 specific antibody (C92 F3 B1) (solid line) or isotype-matched controls (dotted line). The percentage of Hsp70 positively stained cells is shown in the right corner of each graph.

B) Hsp70 cell surface expression was not induced in PBL of healthy human donors following vincristine (V) or paclitaxel (P) treatment.
Untreated (37°C) or heat-shocked (41.8°C) PBL derived from healthy donors (n=5) were treated either with 1μM V or 1μM P and stained with FITC-conjugated Hsp70 antibody C92FB1. The marker indicates the position of the isotype-matched (IgG1) control antibody. The % Hsp70 positively stained cells is shown in the upper right corner of each graph.

Fig 5.

Immunofluorescence staining of tubulin in untreated and cytostatic drug treated K562 cells
Untreated (37°C) or heat-shocked (41.8°C) K562 cells were incubated with 1μM of the cytostatic drugs vincristine (V) or paclitaxel (P). Either untreated (ctrl) or vincristine (V) and paclitaxel (P) treated cells were mounted on glass slides, fixed with 4% paraformaldehyd, incubated with a tubulin specific monoclonal antibody and stained with a secondary FITC-conjugated antibody. Scale bars; 10μm.

Peripheral blood lymphocytes (PBL) were derived from heparinized blood of healthy human volunteers following separation by Ficoll density gradient centrifugation. After washing the cells were counted and resuspended in the same medium that was used for K562 cells.

The examples illustrate the invention.

Example 1: Treatment with cytostatic drugs

Either untreated or heat-shocked tumor cells were incubated with the following antineoplastic agents. With the exception of ifosfamide all agents are available in their active form. The prodrug 4-hydroperoxyifosfamide (4-OOH-IF; kindly provided by ASTA-Medica, Frankfurt, Germany) spontaneously gives rise to pharmacologically equivalent amounts of 4-OH-IF in aqueous solution. Intracellularly, the activated form 4-OH-IF is decomposed to alkylating mustard, when acrolein is split off. For *in vitro* assays the aqueous solution of 4-OOH-IF was freshly prepared directly before each assay (Zalupski and Baker 1988; Multhoff *et al.* 1995b).

The DNA intercalating cytostatic drugs carboplatin (Ribocarbo-L, ribosepharm, München, Germany), doxorubicin (Doxo-cell, cell pharm, Hannover, Germany), fludarabine (Fludara, Schering, Berlin, Germany), cytarabine (Ara-cell, cell pharm, Hannover, Germany), and the tubulin interacting agents vincristine (Vincristin Liquid, Lilly, Giessen, Germany), and paclitaxel (Taxol-100, Bristol, Munich, Germany), were obtained from the pharmaceutical department of the University Hospital Regensburg. All agents were prepared freshly as described for medical applications. For the *in vitro* assays stock solutions of the cytostatic drugs were diluted in cell culture medium. Untreated or heat-shocked cells were incubated at concentrations of 10nM, 1μM, 10μM or 100μM of each drug for 2 hours at 37°C and 5% CO_2. The drug incubation time of 2 hours was chosen in order to study sublethal effects. Furthermore, previously published data from our group indicate that in aqueous solution 4-hydroperoxyifosfamide, the activated form of ifosfamide, gets inactivated within few hours (Multhoff *et al.* 1995b). Following treatment, the cells were washed

twice in ice-cold PBS and then resuspended in fresh culture medium for a recovery period of 2 hours at 37°C.

Example 2: Preparation of cytoplasmic and plasma membrane fractions

Following treatment, cytoplasmic fractions were prepared from 5 x 10^6 cells by incubation of PBS washed cell pellets in 10 mM Tris-buffered saline (pH 7.5) containing 1% Nonidet P-40 (NP-40; Sigma) and 1 mM phenyl-methyl-sulphonyl-fluoride (PMSF; Sigma) as described previously (Botzler *et al.* 1999). Plasma membrane fractions were prepared by a modified method described by Weissman (1991). Briefly, 50 x 10^6 cells were broken by 30 strokes with a tight pestle in a Dounce homogenizer. The cytoplasmic fraction was separated from the membrane fraction by ultracentrifugation at 100,000 g at 4°C. Membrane-bound proteins were separated by treatment of the last pellet with Triton X-100 (Sigma) followed by centrifugation of the unsoluble material at 10,000 g.

Example 3: Definition of nontoxic concentrations of antineoplastic agents on tumor cells

The myelogenous tumor cell line K562 (CCL243, ATCC) and the human colon carcinoma cell line CX2 (see below; TZB 61005, Tumorbank DKFZ, Heidelberg, Germany) (Multhoff *et al.* 1997) were grown in RPMI 1640 medium (GibcoBRL, Eggenstein, Germany) supplemented with 10% heat-inactivated fetal calf serum (FCS, BioWhittaker, Walkersville, Maryland, USA), 6 mM L-glutamine, and antibiotics (100IU/ ml penicillin and 100μg/ ml streptomycin, GibcoBRL, Eggenstein, Germany). In order to obtain exponential growth the cell density was maintained at 0.5 x 10^6.

In order to define the effects of different antineoplastic agents on the viability of either untreated or heat-shocked K562 tumor cells, a 2 hour incubation of the cells with cytarabine (C), ifosfamide (I), vincristine (V), and paclitaxel (P) at the following concentrations 10 nM, 1μM, 10μM, 100μM was performed. Cell viability was evaluated either by trypan blue (GibcoBRL, Eggenstein, Germany) exclusion assays or propidium iodide (PI; Sigma, Munich, Germany) incorporation assays, directly before the preparation of cellular extracts and before flow cytometric analysis. Cells were incubated for 1 min with trypan blue or PI and then analysed. Only dead cells incorporate either trypan blue or PI. Following incubation with PI a minimum of 5000

cells was analysed on a FACSCalibur flow cytometer (Becton Dickinson, Heidelberg, Germany).

As shown in Table 1, a concentration of 100μM of the antineoplastic drugs negatively affects the viability of the cells. The reduction in cell viability at 100μM was less pronounced if the cells were treated with a nonlethal heat shock (41.8°C) for 2 hours before the incubation with the cytostatic drugs. (Briefly, exponentially growing tumor and normal cells were treated at a nonlethal temperature of 41.8°C for 2 hours in a temperature-controlled waterbath (GFL, Burgwedel, Germany) followed by a recovery period of 15 hours at 37°C. Under these conditions the cell viability was greater 99%.) The concentrations, 10nM, 1μM, and 10 μM of the antineoplastic drugs, either under physiological conditions or combined with heat, have been determined as nontoxic for K562 cells.

The effects of the different antitumor agents on apoptotic cell death was determined by annexin staining. Briefly, cells were washed twice in Hepes buffer containing 5 mM $CaCl_2$ and incubated with Annexin-V-FLUOS (Roche) for 10 min at room temperature. Annexin positively stained cells were measured in a FACSCalibur flow cytometer. At a concentration of 1μM none of the drugs induces significant apoptosis in either untreated (37°C) or heat-shocked (41.8°C) K562 cells 24 hours following treatment. As shown in Figure 1A the amount of apoptotic cells following heat shock (41.8°C) was not significantly different from that of untreated (37°C) control cells, 11% versus 5%, respectively. After treatment with the different cytostatic drugs the amount of cells that underwent apoptosis was always below 12%. Therefore, it can be concluded that the concentration of 1μM neither induces necrotic nor apoptotic cell death. Camptothecin was used as a positive control for apoptosis induction. Incubation of K562 cells with camptothecin at a concentration of 2μg/ ml for 4 hours significantly increases the percentage of annexin positive stained up to 51% (Fig. 1B). Stock solutions of camptothecin (5mg/ ml) were diluted in DMSO and stored at 4°C in the dark. A treatment of K562 cells with 100μM of the different cytostatic drugs induces apoptosis in a comparable range of 30% up to 70%.

Example 4: Effects of antineoplastic agents on cytoplasmic Hsp70 levels in K562 cells

To test the inducibility of Hsp70 in K562 cells by antineoplastic drugs, cells maintained under physiological conditions (37°C) or following nonlethal heat shock (41.8°C), were treated with cytarabine (C), the activated form of ifosfamide (I), vincristine (V), or paclitaxel (P) at the nontoxic concentrations 10nM, 1μM, 10μM and at the lethal concentration of 100μM. After a 2 hour recovery period at 37°C, cell lysates were subjected to a 10% SDS-PAGE. Following Western blot analysis using the Hsp70 specific MoAb the cytoplasmic amount of Hsp70 was measured by comparative laser scan densitometry. Specifically, equal protein amounts (5μg or 10μg) derived from freshly prepared cell lysates or plasma membrane fractions were separated electrophoretically on a 10% SDS-PAGE (Laemmli 1970). Following SDS-PAGE the proteins were transferred onto nitrocellulose membrane (PALL Corporation, Ann Arbor, USA) according to a standard protocol of Towbin (1979). Nonspecific binding to nitrocellulose was blocked with 5% skim milk in PBS with 0.5% Tween at room temperature for 30 min. Then the membranes were incubated with an anti-Hsp70 specific monoclonal antibody (clone 7F4, diluted 1 : 1000, kindly provided by Dr. Kremmer, GSF-Munich) and with a secondary antibody (goat anti-rat IgG peroxidase conjugated, dilute 1 : 2000, Dako, Hamburg, Germany), each for 1 hour. Immune complexes were detected using the ECL Western blotting detection system (Amersham, Braunschweig, Germany). Protein bands were scanned and quantified using the ImageMaster 1D Elite Version 3.00 (Amersham Pharmacia Biotech, Braunschweig, Germany). In order to ensure that equal protein amounts have been subjected to the gel, the blots were stained with the antibody CP01 (Oncogene, Boston MA, USA) directed against actin.

As shown in Figure 2A, under physiological temperatures (37°C), following treatment of K562 cells with cytarabine (C) or with ifosfamide (I), the cytoplasmic Hsp70 levels remained unaltered at any of the tested concentrations ranging from 10nM up to 100μM. Identical results were achieved with other DNA or RNA interacting agents, including carboplatin (O'Dywer *et al.* 2000), doxorubicin (DeBeer *et al.* 2001) or fludarabine (Johnson 2000; Grant 1998) (data not shown). Doxorubicin is a member of the anthracycline family that intercalates with DNA and supports uncoiling of

double-strand helices. Fludarabine is cytotoxic for slowly replicating cells since it is incorporated into DNA during strand breaks repair. In contrast to the results obtained with DNA interacting reagents, both tubulin interacting agents, vincristine (V) and paclitaxel (P), result in increased cytoplasmic Hsp70 levels at the nonlethal concentration of 1μM. For vincristine (V) the increase was 38% and for (P) it was 34%. At a concentration of 100μM no increase in cytoplasmic Hsp70 has been observed. This finding is due to the fact that a concentration of 100μM of vincristine (V) reduces cell viabilty to 28% and of paclitaxel (P) to 71% (Table 1).

In addition to K562 cells, the colon carcinoma cells CX2 also was treated with the different cytostatic drugs at the nonlethal concentration of 1μM. Similar to K562 cells, cytarabine (C) and ifosfamide (I) did not influence the cytoplasmic amount of Hsp70, whereas, vincristine (V) and paclitaxel (P), both increase the cytoplamic amount of Hsp70 in the range of 20 to 25%.

Furthermore, the question as to whether a combined treatment consisting of nonlethal heat plus antineoplastic agents exhibits a synergistic effect on the cytoplasmic Hsp70 levels was posed. In line with previously published data (Botzler *et al.* 1999), it could be shown that an exposure of K562 cells to the sublethal temperature of 41.8°C results in an 1.3 up to 2.5 fold increase in the amount of cytoplasmic Hsp70. A combined treatment consisting of heat plus chemotherapy revealed that none of the DNA interacting agents including cytarabine (C), ifosfamide (I), carboplatin (A), doxorubicin (D), and fludarabine (F), did alter Hsp70 levels as compared to heat shock alone. In Figure 2B the relative amount of cytoplasmic Hsp70 following nonlethal heat together with the corresponding Western blot was shown for cytarabine (C), ifosfamide (I), vincristine (V), and paclitaxel (P). In contrast to the DNA interacting agents cytarabine (C) and ifosfamide (I), heat plus vincristine (V) or heat plus paclitaxel (P), both result in an additive elevation in the cytoplasmic amount of Hsp70 (31% and 46%, respectively) (Fig. 2B). The data shown in Figures 2A and 2B represent one experiment out of at least three that exhibit comparable results.

Example 5: Effects of antineoplastic agents on the amount of membrane-bound Hsp70 on tumor cells

Beside its chaperoning function, extracellular localized Hsp70 is known to stimulate NK cell activity. Cell membrane-bound Hsp70 acts as a tumor-selective target recognition structure (Multhoff *et al.* 1995a; Multhoff *et al.* 1995c). In order to evaluate the immunological effects of a cytarabine (C), ifosfamide (I), vincristine (V), and paclitaxel (P) treatment on tumor cells membrane expression of Hsp70 was investigated. It was investigated whether enhanced cytoplasmic Hsp70 levels correlate with an increased amount of membrane-bound Hsp70. Membrane localization of Hsp70 in either untreated or heat-shocked K562 cells was studied using the nonlethal concentration of 1μM of cytarabine (C), ifosfamide (I), vincristine (V), and paclitaxel (P). Following treatment plasma membrane fractions were prepared and subjected to SDS-PAGE. One representative Western blot analysis out of three using the Hsp70 specific MoAb is shown in Figure 3A. A quantitative laser scan densitometry of the results is summarized in Figure 3B. In accordance with the findings of cytoplasmic Hsp70 levels, vincristine (V) as well as paclitaxel (P), both result in an enhanced Hsp70 membrane expression in untreated and heat-shocked K562 cells. Under physiological conditions the increase in membrane-bound Hsp70 was 23% for vincristine (V), and 24% for paclitaxel (P); following heat shock vincristine (V) results in an Hsp70 increase greater 100% and 43% for paclitaxel (P). Again, cytarabine (C) as well as the activated form of ifosfamide (I) did not affect the amount of membrane-bound Hsp70.

The increase in Hsp70 membrane expression could be confirmed by flow cytometric analysis. Briefly, indirect immunofluorescence studies were performed using the Hsp70 specific monoclonal antibody (MoAb, clone C92 F3 B1, multimmune GmbH, Regensburg, Germany), the MHC class I specific MoAb (W6/32) and isotype-matched control antibodies (IgG1, IgG2a Immunotech, Marseille, France) as primary antibodies and FITC- or PE-conjugated rabbit anti-mouse secondary antibodies (Dako, Hamburg, Germany). Briefly, the cells were incubated with the primary antibodies at 4°C for 30 min. After two washing steps the cells were stained with a secondary antibody for another 30 min at 4°C. A quantitative flow cytometric analysis was performed using a FACSCalibur instrument (Becton Dickinson, Heidelberg, Germany). The percentage of positively stained cells was determined as the number of positively stained cells minus the number of cells stained with an isotype-matched

negative control antibody. Only viable, propidium iodide (PI) negative cells were analysed.

Although the absolute numbers of Hsp70 positive cells and the amount of membrane-bound Hsp70 could not be directly compared, the data obtained using different methods reflect a similar pattern. As shown in Figure 4A, preincubation of K562 cells with vincristine (V) and paclitaxel (P) results in a significant increase in the percentage of viable Hsp70 positive cells, whereas cytarabine (C) and ifosfamide (I) did not affect the percentage of Hsp70 positively cells (data not shown). By comparison with untreated control cells the amount of Hsp70 positive cells was increased from 43% up to 59% by vincristine, and up to 52% by paclitaxel as indicated in the upper right corner of each graph (Fig 4, left side). As shown in Figure 4, right side, comparable results were obtained with pre-heated K562 cells: heat alone enhances the amount of Hsp70 positive cells from 45% up to 51%. Following heat shock and incubation with vincristine (V) or paclitaxel (P), the amount of membrane-bound Hsp70 positive cells is increased from 52% up to 72% and 70%, respectively (Fig 4, right side). Similar results could be obtained with the human colon carcinoma cells CX2.

Example 6: Effects of antineoplastic agents vincristine (V) and paclitaxel (P) on the formation of tubulin aggregates in tumor cells

The data clearly showed that following treatment with nonlethal concentrations of tubulin interacting agents the cytoplasmic and membrane-bound Hsp70 levels were increased. In contrast, DNA interacting agents at identical concentrations did not affect Hsp70 levels. Protein aggregates are known to induce Hsp70 expression (Ait-Aissa *et al.* 1999). Therefore, it was investigated whether a nonlethal concentration of vincristine (V) or paclitaxel (P) induces tubulin aggregation in tumor cells. Fluorescence microscopy was performed using an antibody directed against tubulin following treatment of K562 tumor cells with the nonlethal concentration of 1μM vincristine (V) or 1μM paclitaxel (P). To this effect, tumor cells were treated with 1μM vincristine (V) or paclitaxel (P) for 2 hours. Following a recovery period of 2 hours at 37°C the cells were washed and settled on poly-L-lysine coated glass slides, fixed with 4% paraformaldehyde in PBS for another 30 min at room temperature. After

fixation the cells were incubated with an antibody directed against tubulin (AB-1, Oncogene, Boston, USA) and stained with a goat-anti-mouse FITC conjugated secondary antibody (Dako, Glostrup, Denmark) for another 30 min. The slides were mounted with Fluorescent Mounting Medium (Dako, Carpinteria, USA) and then the samples were analysed for transmission and fluorescence using a Zeiss model Axioscop 2 scanning microscope (Zeiss Jena, Germany) equipped with a 100x (planar) or 63x (apochromatic) oil-immersion objective and standard filters. Section of specific fluorescence were taken; the localization of tubulin and tubulin aggregates was visualized with FITC in green. Images were treated by multiplicative shading correction using software Axiovision (Zeiss Vision, Jena, Germany).

As shown in Figure 5 upper part, untreated control cells (37°C) and heat-shocked cells (41.8°C) exhibit a regular tubulin formation as demonstrated on mitotic cells. Following treatment with tubulin interacting agents nearly no mitotic cells were found and the tubular network was significantly distrubed. Cells treated with 1μM vincristine (V) at physiological temperature conditions frequently show tubulin staples in the nucleus and an irregular tubulin distribution in the cytoplasm (Fig. 5, middle left side). After heat shock the formation of tubulin aggregates in the cytoplasm was even more pronounced (Fig. 5, middle right side). Treatment with paclitaxel (P, 1μM) also induces tubulin aggregates, similar to that seen with vincristine (V) (Fig. 5, lower panels). Under physiological temperatures predominantly tubulin stapels are found in the nucleus (Fig. 5 lower left side), and following heat shock nearly all cells contain clumpy tubular spots in the cytoplasm (Fig. 5 lower right side).

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Table 1

Cell viability of K562 cells following treatment with different neoplastic agents including cytarabine (C), ifosfamide (I), vincristine (V), and paclitaxel (P) at the different concentrations, 10nM, 1μM, 100μM.

The data represent the mean of three independent experiments.

temperature	agent	10nM	1μM	100μM	control
		% viable K562 cells			
37°C	cytarabine (C)	100 ± 0.5	99 ± 1.5	99 ± 0.0	99 ± 0.0
	ifosfamide (I)	100 ± 1.0	97 ± 0.0	98 ± 1.5	99 ± 0.0
	vincristine (V)	99 ± 0.5	96 ± 0.5	28 ± 5.9	99 ± 0.0
	paclitaxel (P)	99 ± 0.0	96 ± 0.0	71 ± 12.1	99 ± 0.0
41.8°C	cytarabine (C)	98 ± 0.5	98 ± 0.0	97 ± 1.0	99 ± 0.1
	ifosfamide (I)	99 ± 0.5	99 ± 0.0	94 ± 5.6	99 ± 0.2
	vincristine (V)	99 ± 0.0	97 ± 0.0	92 ± 2.9	99 ± 0.0
	paclitaxel (P)	99 ± 0.5	100 ± 0.5	91 ± 3.2	99 ± 0.0

Table 2

					Vincristin					Paclitaxel
control	96,0	100,0	98,8	100,0	98,7	100,0	100,0	100,0	100,0	100,0
10 nM	100,0	100,0			100,0	100,0	100,0			100,0
100 nM	100,0	100,0			100,0	100,0	100,0			100,0
200 nM	97,3	97,6			97,5	100,0	100,0			100,0
300 nM	94,5	100,0			97,8	100,0	100,0			100,0
400 nM	92,0	100,0			96,0	98,6	100,0			99,3
500 nM	94,4	100,0			97,2	97,2	98,2			97,7
600 nM	95,0	100,0			97,5	94,7	100,0			97,4
700 nM	96,6	100,0			98,3	85,5	97,2			91,4
800 nM	93,5	100,0			96,8	93,5	98,0			95,8
900 nM	95,8	100,0			97,9	91,8	98,1			95,0
1 µM	89,8	100,0	97,9		95,9	93,5	96,9	80,6		90,3
10 µM	90,8	95,2	97,1		94,4	92,6	93,0	76,0		87,2
20 µM			97,1		97,1			61,4		61,4
30 µM			94,4		94,4			61,7		61,7
40 µM			91,8		91,8			53,3		53,3
50 µM			94,0		94,0			48,8		48,8
60 µM			96,2		96,2			31,8		31,8
70 µM			93,5		93,5			23,8		23,8
80 µM			95,5		95,5			14,5		14,5
90 µM			90,6		90,6			5,2		5,2
100 µM	82,2	61,1	80,5	90,2	78,5	0,0	10,0	4,2	9,2	5,9
150 µM				90,0	90,0				3,1	3,1
200 µM				79,4	79,4				1,2	1,2
250 µM				72,9	72,9				0,0	0,0
300 µM				26,3	26,3				0,0	0,0

Definition of the sublethal concentration range of vincristin (100nM - 250µM) and paclitaxel (100nM - 20µM)

Claims

1. Use of a drug inducing intracellular protein aggregation for the preparation of a pharmaceutical composition wherein the drug is in a dose of 135-175 mg/m^2 body surface for the treatment of a tumor or a bacterial or viral infection.

2. The use of claim 1 wherein said drug is vincristine.

3. The use of claim 1 wherein said drug is paclitaxel.

4. The use of any one of claims 1 to 3 wherein said tumor is a malign tumor.

5. The use of claim 4 wherein said malign tumor is a tumor selected from the following: Colorectal, pancreas, melanoma, lung carcinoma (small and non-small), ovarian, kidney, mammary, head and neck, stomach, liver, oesophagus, prostata carcinomas, sarcomas, myeloproliferative diseases, non-Hodgkin's lymphoma (NHL), AML (acute myelocytic leukemia), acute lymphocytic leukemia (ALL), multiple myeloma, chronic myelocytic leukemia (CML) and chronic lymphocytic leukemia (CLL), B- and T-lymphomas, CNS lymphomas, gastrointestinal lymphomas, and cutaneous lymphomas.

6. A method of treating a patient suffering from a tumor or a bacterial or viral infection comprising administering to said patient a drug inducing intracellular protein aggregation in a dose of 135-175 mg/m^2 body surface.

7. The method of claim 6 wherein said drug is vincristine.

8. The method of claim 6 wherein said drug is paclitaxel.

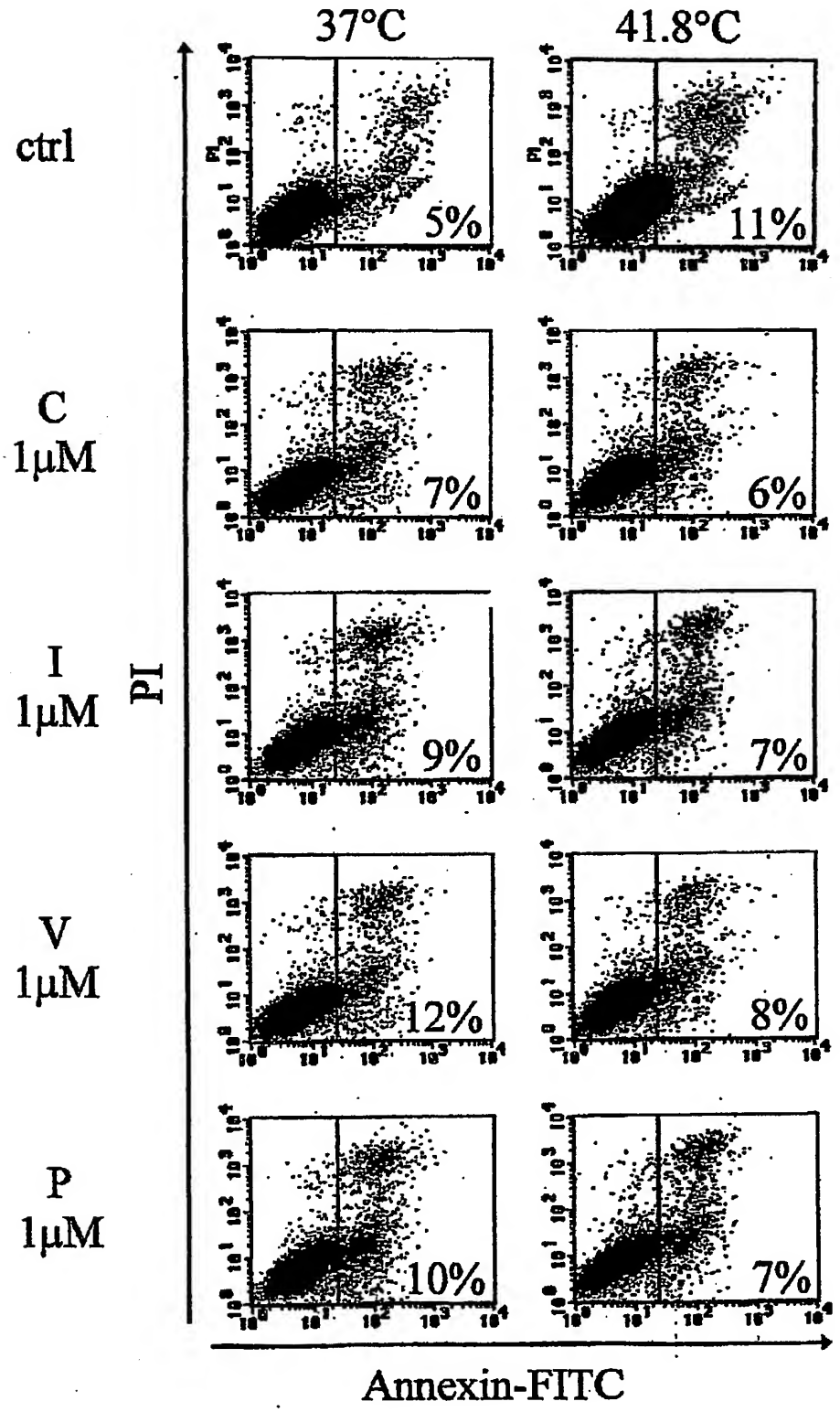
9. The method of any one of claims 6 to 8 wherein said tumor is a malign tumor.

10. The method of claim 9 wherein said malign tumor is a tumor selected from the following: Colorectal, pancreas, melanoma, lung carcinoma (small and non-small), ovarian, kidney, mammary, head and neck, stomach, liver, oesophagus, prostata carcinomas, sarcomas, myeloproliferative diseases, non-Hodgkin's lymphoma (NHL), AML (acute myelocytic leukemia), acute lymphocytic leukemia (ALL), multiple myeloma, chronic myelocytic leukemia (CML) and chronic lymphocytic leukemia (CLL), B- and T-lymphomas, CNS lymphomas, gastrointestinal lymphomas, and cutaneous lymphomas.

11. The method of any one of claims 6 to 10 further comprising the administration of heat in the range between > 37°C to 43°C for 1 to 12 hours to said patient.

12. A pharmaceutical composition comprising a drug inducing intracellular protein aggregation in a dose of 135-175 mg/m^2 body surface.

Figure 1A


37°C
41.8°C
ctrl
C
1μM
I
1μM
V
1μM
P
1μM
5%
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7%
6%
9%
7%
12%
8%
10%
7%
PI
Annexin-FITC

Figure 1B

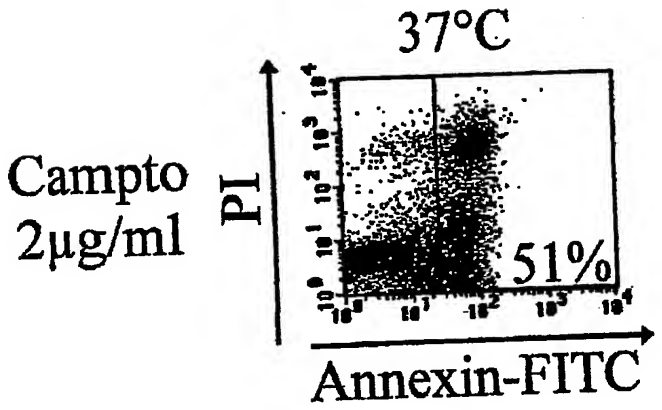

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Figure 2A

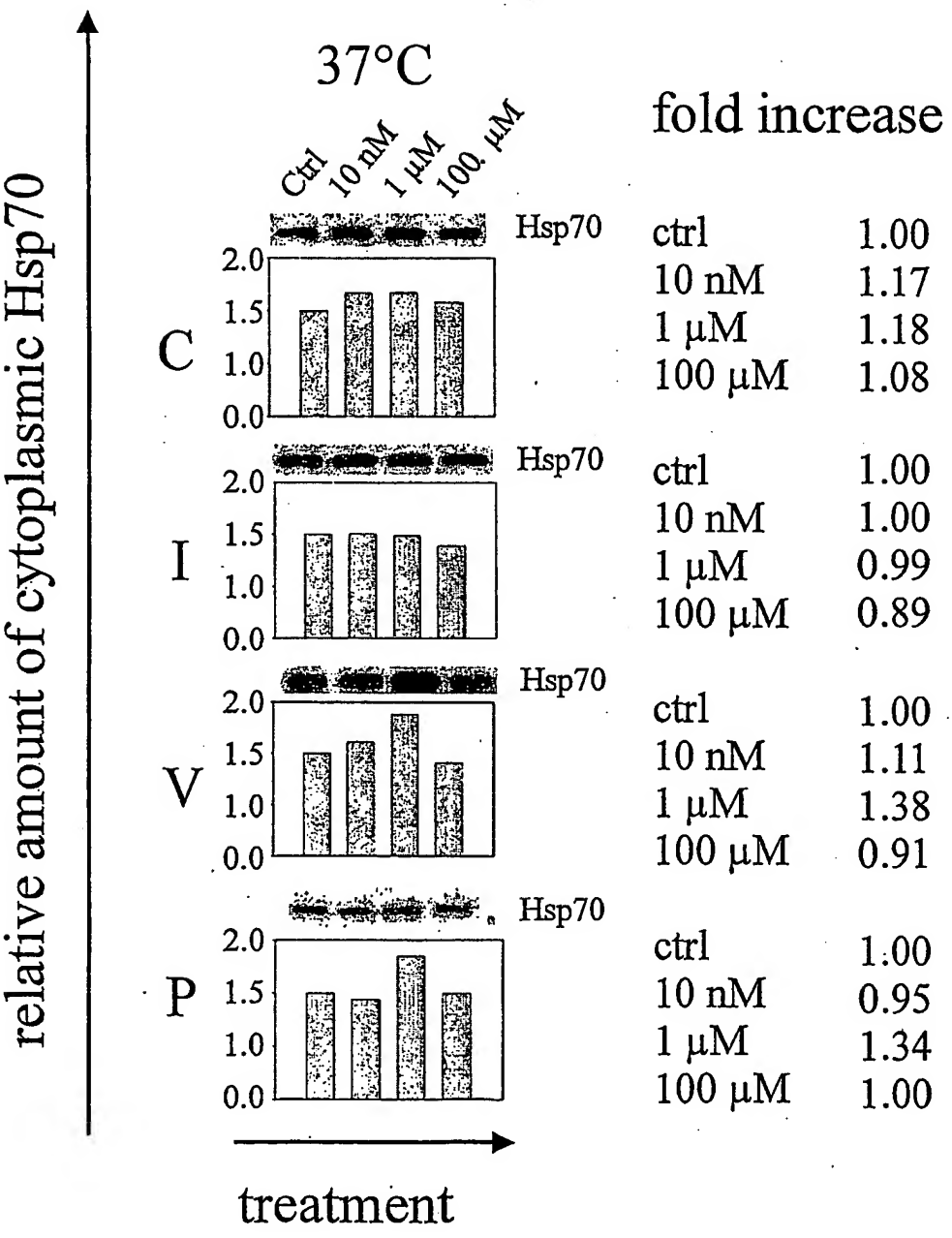

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Figure 2B

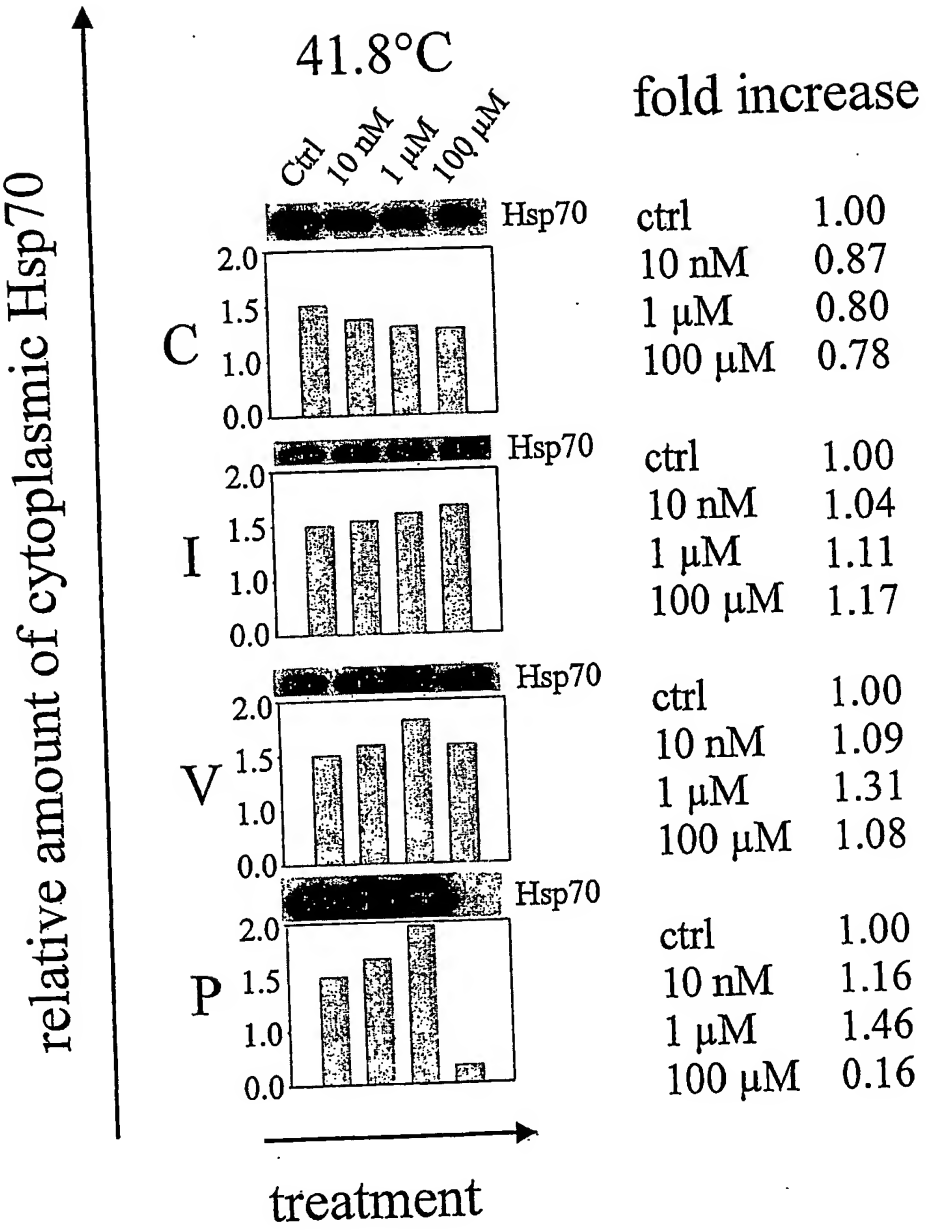

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Figure 2C

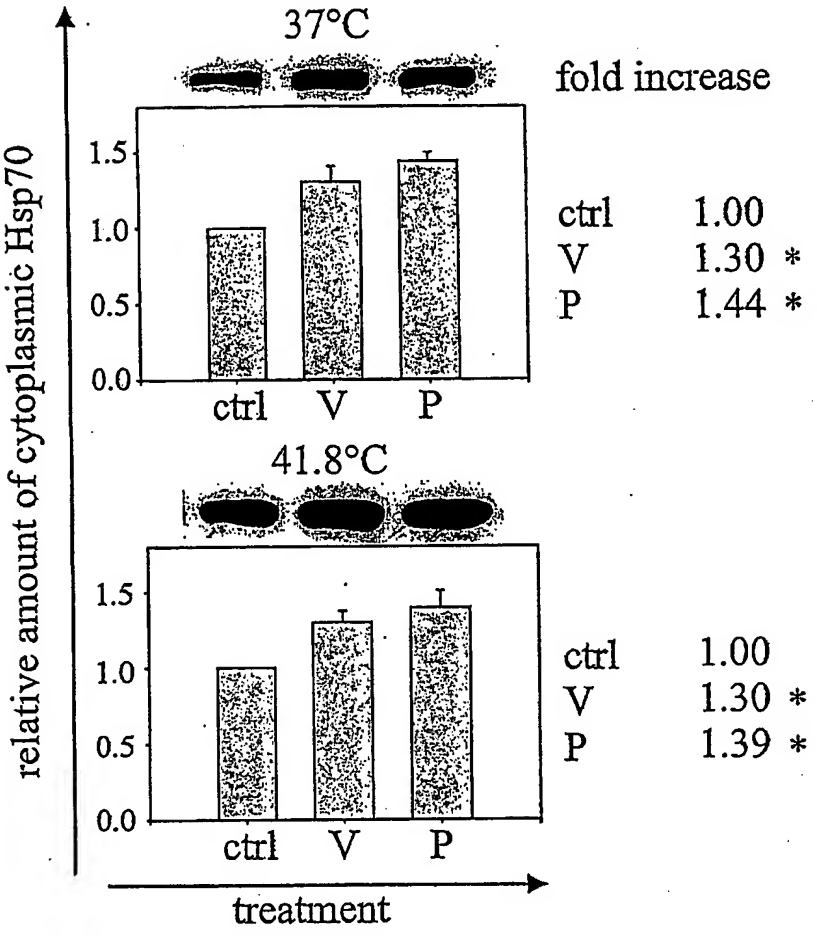

Figure 3A

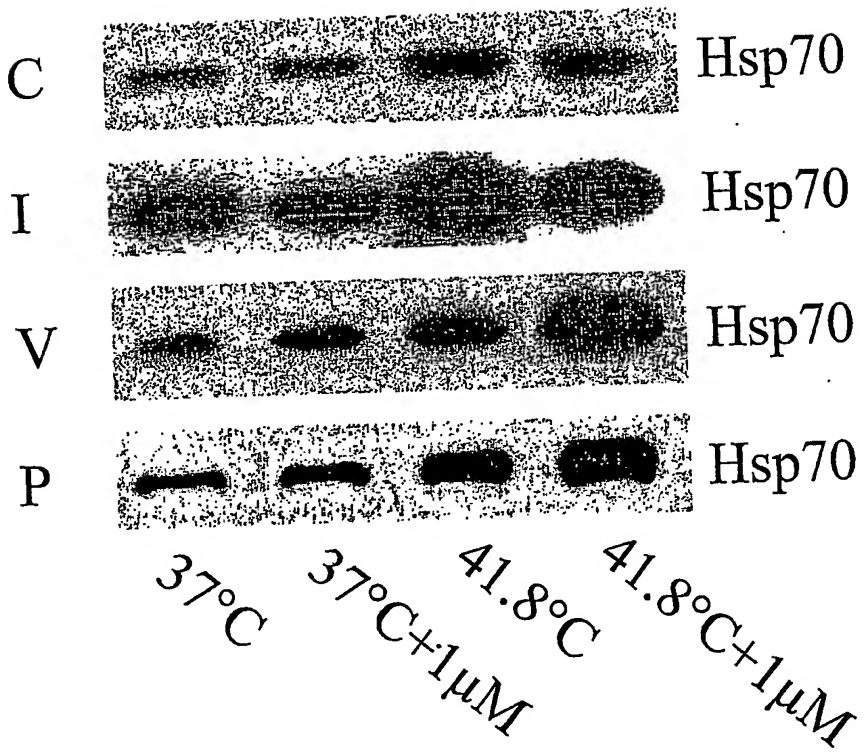

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Figure 3B

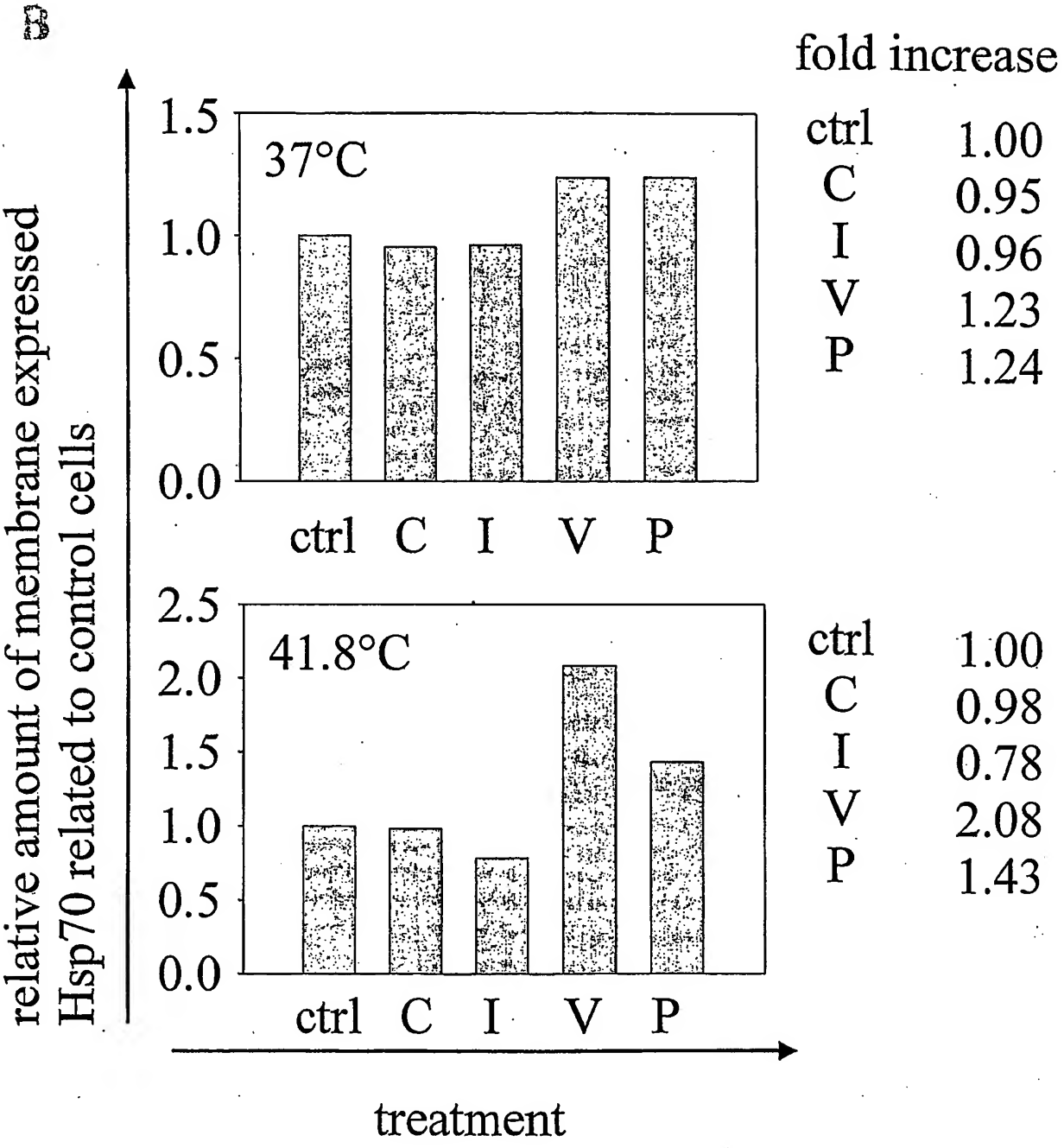


Figure 4A

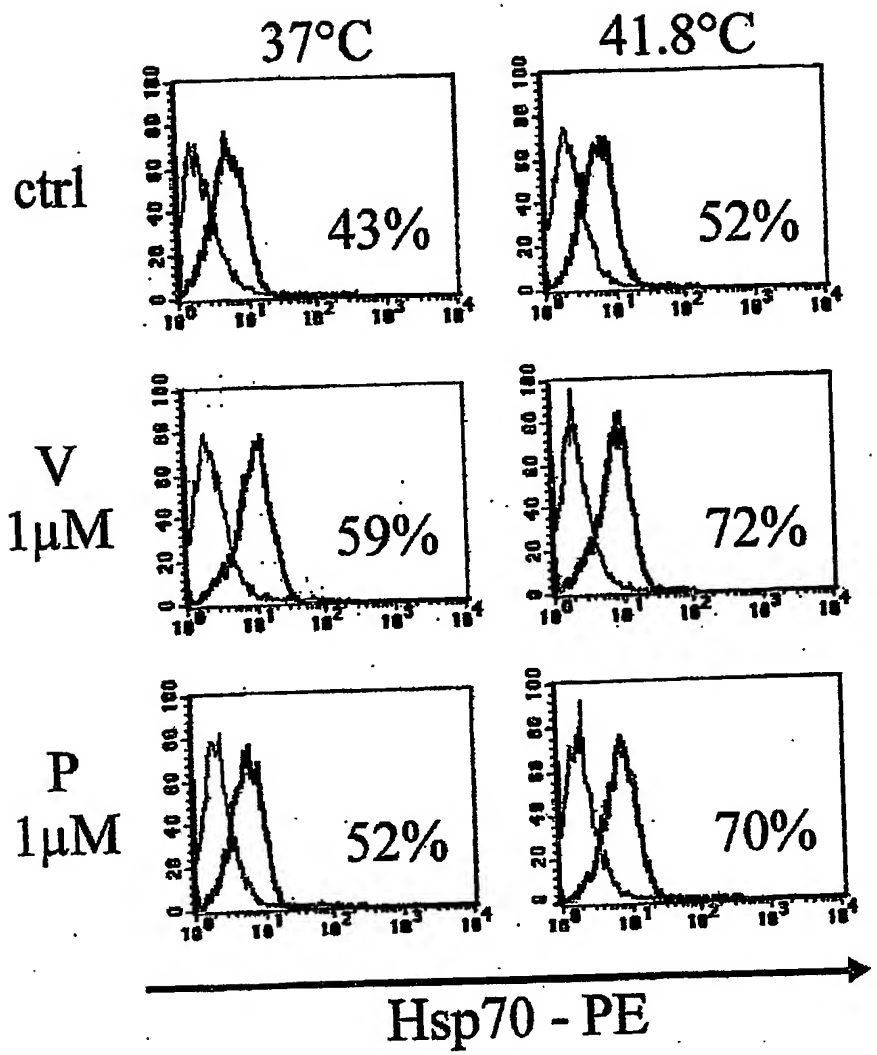


Figure 4B

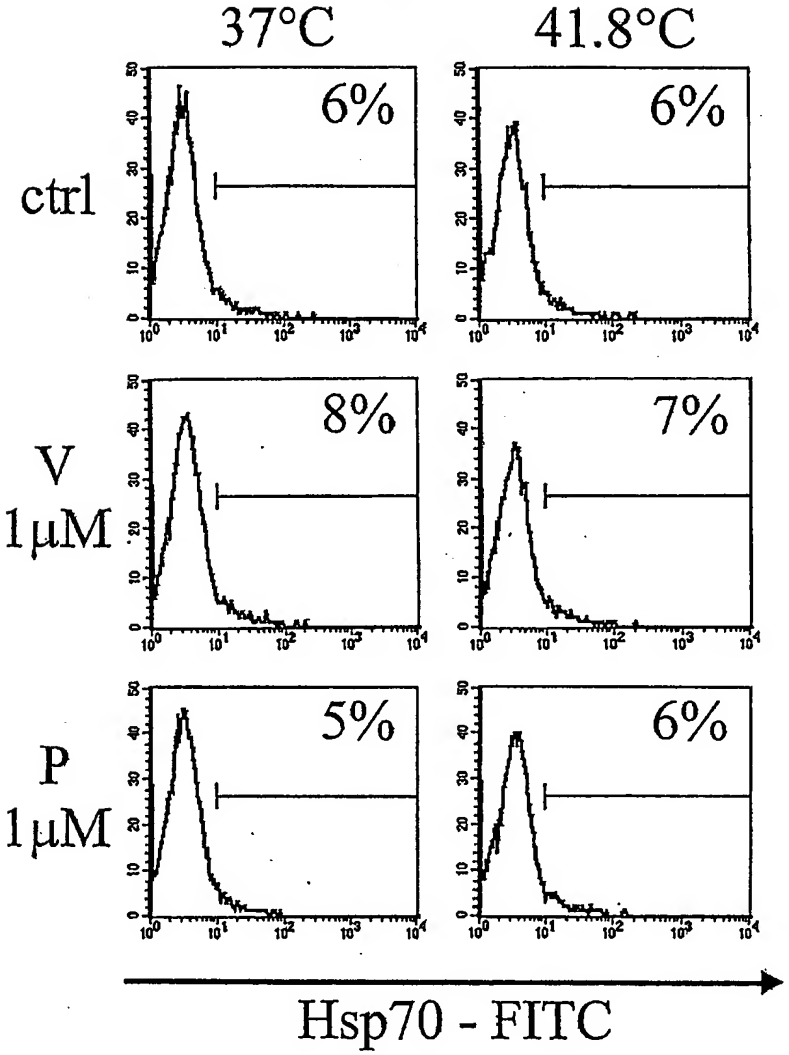
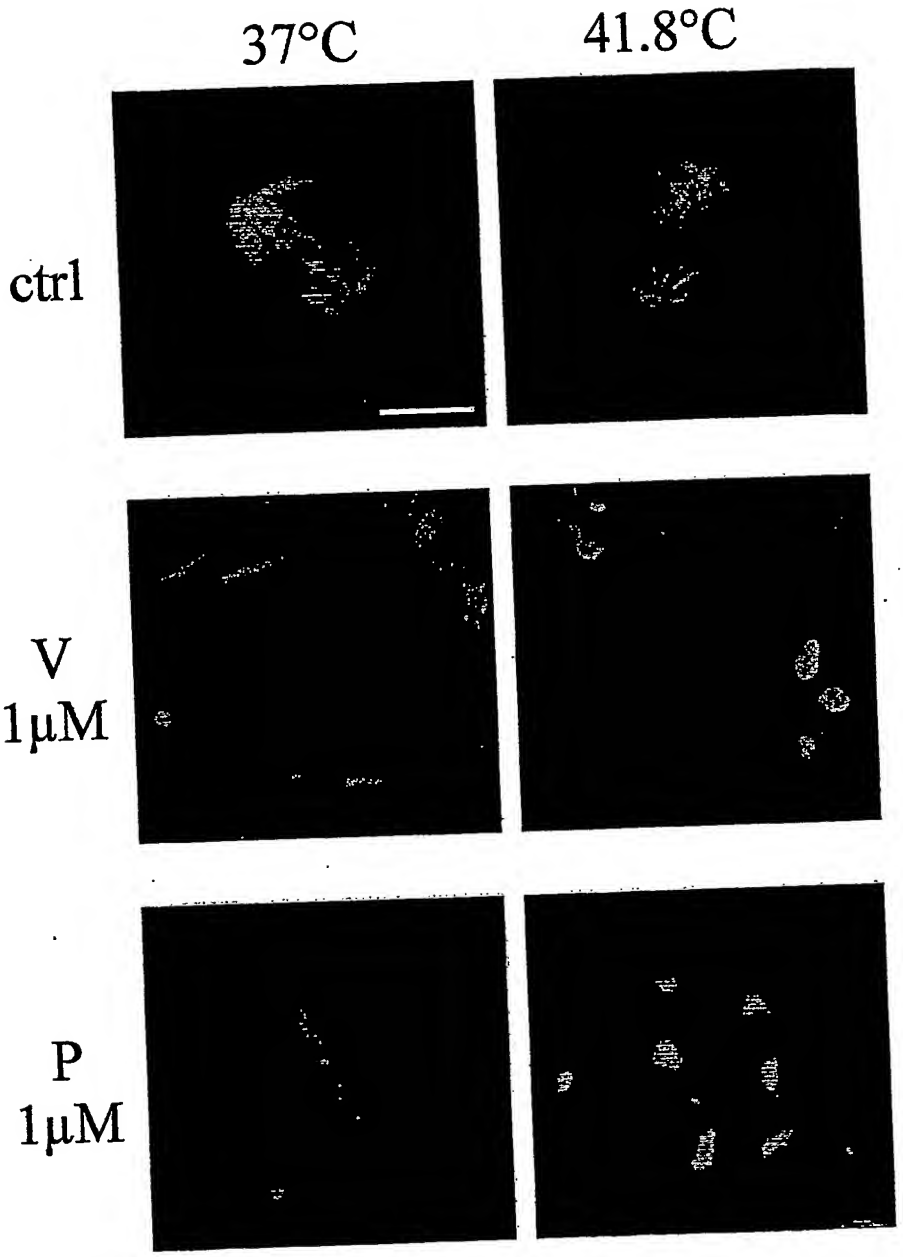


Figure 5



INTERNATIONAL SEARCH REPORT

PCT/EP 03/03785

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A61K31/337 A61K31/475 A61P35/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, PAJ, BIOSIS, MEDLINE, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	PARFITT K: "Martindale 32nd ed." 1999 , PHARMACEUTICAL PRESS , LONDON, UK XP002250751 page 570-571 ---	2,7
A	MCCUNE J S ET AL: "Appropriateness of maximum-dose guidelines for vincristine." AMERICAN JOURNAL OF HEALTH-SYSTEM PHARMACY: AJHP: OFFICIAL JOURNAL OF THE AMERICAN SOCIETY OF HEALTH-SYSTEM PHARMACISTS. UNITED STATES 1 AUG 1997, vol. 54, no. 15, 1 August 1997 (1997-08-01), pages 1755-1758, XP009015376 ISSN: 1079-2082 page 1758, paragraph entitled "Conclusion" --- -/--	2,7

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
8 August 2003	29/08/2003

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Borst, M

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	EP 0 783 885 A (SQUIBB BRISTOL MYERS CO) 16 July 1997 (1997-07-16) claims 1, 2, 7	1,3-6, 8-11 11
Y	OTHMAN TIMOTHY ET AL: "Hyperthermic enhancement of the apoptotic and antiproliferative activities of paclitaxel." PHARMACOLOGY (BASEL), vol. 62, no. 4, May 2001 (2001-05), pages 208-212, XP009015375 ISSN: 0031-7012 page 210-212, paragraph entitled "Discussion"	11
Y	CIVIDALLI A ET AL: "Hyperthermia enhances the response of paclitaxel and radiation in a mouse adenocarcinoma" INTERNATIONAL JOURNAL OF RADIATION ONCOLOGY BIOLOGY PHYSICS 1999 UNITED STATES, vol. 44, no. 2, 1999, pages 407-412, XP002250750 ISSN: 0360-3016 page 410-411, paragraph entitled "Discussion"	11

INTERNATIONAL SEARCH REPORT

PCT/EP 03/03785

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.: –
because they relate to subject matter not required to be searched by this Authority, namely:

Although claims 6-11 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition (Rule 39.1(iv) PCT).

2. [X] Claims Nos.: 1(part),2,3(part)-6(part),7,8(part)-11(part),12
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

see FURTHER INFORMATION sheet PCT/ISA/210

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Claims Nos.: 1(part),2,3(part)-6(part),7,8(part)-11(part),12

1. Clarity (Article 6 PCT)
Present independent claim 12 relates to a pharmaceutical composition the drug content of which is defined in terms of a dose of 135-175 mg/m2 body surface. As the body surface depends on the individual patient to be treated with the composition and the application does not contain any information in this respect, it is not clear, how much of the drug is contained in the pharmaceutical composition according to claim 12 on file. The claim lacks so clarity that a meaningfull search is not possible.

2. Disclosure (Article 5 PCT)
2.1. Claims 2 and 7 on file relate to the treatment of tumour patients with vincristine at a dose of 135-175 mg/m2.. However, as it is known from D1 (page 570-571) or D2 (page) the maximum tolerated dose for vincristine is at mg/m2. Thus, the dose claimed exceeds the maximum tolerated dose for vincristine by factor , resulting in severe neurotoxic side effects, if not death of the patient. In view of the toxic or even letal dose of vincristine it is not possible to put the invention according to claims 2 and 7 into practice.
2.2. Moreover, present independent claims 1 and 6 relate to a "drug inducing intracellular protein aggregation". The application on file provides two embodiments for said drugs, ie. paclitaxel and vincristine. As set out under item 2.1. above, for vincristine it is not possible to carry out the invention on file. Therefore, in case it was possible to identify further intracellular protein aggregating drugs, when following the instructions given in the description (page 5, line 5-24), it would have to be concluded in view of the largely differing dose ranges of individual drugs that the dose of 135-175 mg/m2 body surface as defined in claims 1 and 6 will only in exceptional cases be appropriate for solving the problem of the present application (page 3, line 26-29), which is to provide an efficacious treatment of tumours and infections that is not accompagnied by adverse effects. Thus, the skilled person, when seeking to identify further embodiments of the drugs claimed, is confronted with the additional and undue burden of verifying whether an individual drug at the dose of 135-175 mg/m2 as claimed will solve the problem of the application on file or not.
2.3. In conclusion, the application lacks so disclosure that a meaningful search for claims 1 to 11 on file is possible for those parts only which refer to the treatment of a tumour with paclitaxel in a dose of 135-175 mg/m2 body surface.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

receipt of the search report or during any Chapter II procedure.

INTERNATIONAL SEARCH REPORT

PCT/EP 03/03785

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP 0783885 A	16-07-1997	EP 0783885 A2	16-07-1997
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(74) Agent: **STEINECKE, Peter**; Knauthe Eggers, Rechtsanwälte Patentanwälte, P.O. Box 31 01 40, 80102 Munich (DE).

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(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PT, RO, SE, SI, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report*

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(15) Information about Correction:
see PCT Gazette No. 07/2004 of 12 February 2004, Section II

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: NON-TOXIC AMOUNTS OF PROTEIN-AGGREGATING SUBSTANCES STIMULATE HSP70 EXPRESSION AND FUNCTION AS ANTI-TUMOR AGENTS

(57) Abstract: The present invention relates to the use of a drug inducing intracellular protein aggregation for the preparation of a pharmaceutical composition wherein the drug is in a dose of 135-175 mg/m2 body surface for the treatment of a tumor, a bacterial infection or a viral infection. Preferably said drug is selected from vincristine and paclitaxel. The present invention further relates to a method, of treating a patient suffering from a tumor, a bacterial infection or a viral infection comprising administering to said patient a drug inducing intracellular protein aggregation in a dose of 135-175 mg/m^2 body surface. It is preferred that said drug is administered in combination with heat treatment.

WO 2003/086383 A1